FORM PTO-1390 (REV 1-98) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 5722-2

U S APPLICATION NO (If known, see 37 C F R 1 5): 09/446402

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US98/13093	June 24, 1998	June 25, 1997

TITLE OF INVENTION

COMPOSTIONS AND METHODS FOR ACTIVATING GENES OF INTEREST

APPLICANT(S) FOR DO/EO/US

Charles Allen Black, Jr.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. To 16. Below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3 31 is included.

13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information: Small Entity Statement; Statement in Support of Sequence Listing; hard copy Sequence Listing and computer diskette with Sequence List

U.S. APPLICATION NO (If known, see 37 CFR 1.5) 09/446402	INTERNATIONAL APPLICATION NO PCT/US98/13093	ATTORNEY'S DOCKET NUMBER 5722-2

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$970.00**		
International preliminary examination fee (37 CRF 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$840.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search (37CFR 1 445(a)(2)) paid to USPTO **$760.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO But all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$ 96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$ 96.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	**$**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	15 -20 =	0	**X $18.00**	**$ 0.00**	
Independent Claims	4 - 3 =	1	**X $78.00**	**$ 78.00**	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			**+ $260.00**	**$**	
TOTAL OF ABOVE CALCULATIONS =				**$ 174.00**	
Reduction by ½ for filing by small entity, if applicable. A Small Entity statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				**$ 87.00**	
SUBTOTAL =				**$ 87.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				**$**	
TOTAL NATIONAL FEE =				**$ 87.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				**$**	
TOTAL FEES ENCLOSED =				**$ 87.00**	
				Amount to be Refunded	**$**
				Charged	**$**

a. ☒ A check in the amount of $ 87.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 16-0605 in the amount of $ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-0605. A duplicate copy of this sheet is enclosed.

Note: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO: W. Murray Spruill

SIGNATURE
REGISTRATION NUMBER 32,943
ALSTON & BIRD LLP
Post Office Drawer 34009
Charlotte, NC 28234
Tel. Charlotte Office (704) 331-6000
Fax Charlotte Office (704) 334-2014

"Express Mail" Mailing Label Number EL247264725US
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Marilyn Eldridge

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney's Docket No. 5722-2

Applicant:	Charles Allen Black, Jr.
Application No.	To be assigned
Filed:	Concurrently herewith
Title:	COMPOSITIONS AND METHODS FOR ACTIVATING GENES OF INTEREST

STATEMENT CLAIMING SMALL ENTITY STATUS
(37 C.F.R. § 1.9(f) and 1.27(b))--INDEPENDENT INVENTOR

As a below named inventor, I hereby state that I qualify as an independent inventor as defined in 37 C.F.R. § 1.9(c) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office described in:

☒ the specification filed herewith with title as listed above.

☐ the application identified above.

☐ the patent identified above.

I have not assigned, granted, conveyed, or licensed, and am under no obligation under contract or law to assign, grant, convey, or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or nonprofit organization under 37 C.F.R. § 1.9(e).

Each person, concern, or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

☒ No such person, concern, or organization exists.

☐ Each such person, concern, or organization is listed below.

FULL NAME: _____
ADDRESS: _____
☐ INDIVIDUAL ☐ SMALL BUSINESS ☐ NONPROFIT ORGANIZATION

FULL NAME: _____
ADDRESS: _____
☐ INDIVIDUAL ☐ SMALL BUSINESS ☐ NONPROFIT ORGANIZATION

Separate statements are required from each named person, concern, or organization having rights to the invention stating their status as small entities. (37 C.F.R. § 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. § 1.28(b))

Charles Allen Black, Jr.

Signature of Inventor)

11/24/99

Date

09/446402

Attorney's Docket No. 5722-2

PATENT

420 Rec'd PCT/PTO 20 DEC 1999

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Charles Allen Black, Jr.
Appl. No.: To be assigned
Filed: Concurrently herewith
For: COMPOSITIONS AND METHODS FOR ACTIVATING GENES OF INTEREST

STATEMENT IN SUPPORT OF FILING A SEQUENCE LISTING UNDER 37 CFR § 1.821(f)

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I hereby state that the content of the paper Sequence Listing set forth on pages 21-29 of the Specification and computer readable copies of the Sequence Listing, submitted concurrently herewith in accordance with 37 CFR § 1.821(c) and (e), are the same.

Respectfully submitted,

W. Murray Spruill
Attorney for Applicant
Registration No. 32,943

ALSTON & BIRD LLP
Post Office Drawer 34009
Charlotte, NC 28234
Tel Raleigh Office (919) 420-2200
Fax Raleigh Office (919) 420-2260

"Express Mail" Mailing Label Number EL247264725US Date of Deposit: December 20, 1999 I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Box Patent Application, Assistant Commissioner of Patents, Washington, DC 20231. Marilyn Eldridge	**CERTIFICATE OF MAILING** I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner For Patents, Washington, DC 20231, on _____

COMPOSITIONS AND METHODS FOR ACTIVATING GENES OF INTEREST

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/050,772, filed June 25, 1997.

FIELD OF THE INVENTION

The present invention relates to methods and compositions for activating genes of interest particularly in the presence of a target gene.

BACKGROUND OF THE INVENTION

The nature of and basic approaches to cancer treatment are constantly changing. At present, adjuvant chemotherapy routinely follows local treatment of cancers. Clinical protocols are now exploring genetic therapies, manipulations of the immune system, stimulation of normal hematopoietic elements, induction of differentiation in tumor tissues, and inhibition of angiogenesis. Research in these new areas has led to applications for nonmalignant disease.

At the same time, the new clinical protocols have a narrow therapeutic index as well as a great potential for causing harmful side effects. A thorough understanding of the pharmacology, drug interactions, and clinical pharmacokinetics is essential for safe and effective use in human beings.

The therapy of viral infection is in its infancy. Bacterial infection is typically treated with agents, such as antibiotics, which take advantage of the differences in metabolism between the infecting organism and its host. However, viruses largely employed the host's own enzymes to effect the replication, and thus leave few opportunities for pharmacological intervention. By employing strong regulatory elements, the virus obtains transcription and translation of its own genes at the expense of host genes.

In mammals, viral infection is combatted naturally by cytotoxic T-lymphocytes, which recognize viral proteins when expressed on the surface of host cells, and lyse the infected cells. Destruction of the infected cell prevents

the further replication of the virus. Other defenses include the expression of interferon, which inhibits protein synthesis and viral budding, and expression of antibodies, which remove free viral particles from body fluids. However, induction of these natural mechanisms require exposure of the viral proteins to the immune system. Many viruses exhibit a dormant or latent phase, during which little or no protein synthesis is conducted. The viral infection is essentially invisible to the immune system during such phases.

Retroviruses carry the infectious form of their genome in the form of a strand of RNA. Upon infection, the RNA genome is reverse-transcribed into DNA, and is typically then integrated into the host's chromosomal DNA at a random site. On occasion integration occurs at a site which truncates a gene encoding an essential cellular receptor or growth factor, or which places such a gene under control of the strong viral cis-acting regulatory element, which may result in transformation of the cell into a malignant state.

Viruses may also be oncogenic due to the action of their trans-acting regulatory factors on host cell regulatory sequences. In fact, oncogenesis was the characteristic which led to the discovery of the first known retroviruses to infect humans. HTLV-I and HTLV-II (human T-lymphotrophic viruses I and II) were identified in the blood cells of patients suffering from adult T-cell leukemia (ATL), and are believed to induce neoplastic transformation by the action of their transactivating factors on lymphocyte promoter regions. Two additional retroviruses have been found to infect humans. These viruses, HIV-I and HIV-II, are the etiological agents AIDS.

Current therapy for HIV infection includes new drugs called protease inhibitors. These drugs can dramatically reduce HIV levels in the blood when taken with other antiviral compounds such as AZT. At the same time, natural weapons in the immune systems's defenses polypeptide molecules called chemokines, have been unveiled as potent foes of HIV.

Antisense oligodeoxynucleotides have been proposed as a major class of new pharmaceuticals. In general, antisense refers to the use of small, synthetic oligonucleotides resembling single-stranded DNA, to inhibit gene expression. Gene expression is inhibited through hybridization to coding (sense) sequences in

a specific messenger RNA (mRNA) target by Watson-Crick base pairing in which adenosine and thymidine or guanosine and cytidine interact through hydrogen bonding.

Following the simple base-pairing rules which govern the interaction between the antisense oligodeoxynucleotides and the cellular RNA, allow the design of molecules to target any gene of a known sequence. A major advantage of this strategy is the potential specificity of action. In principal, an antisense molecule can be designed to target any single gene within the entire human genome, potentially creating specific therapeutics for any disease in which the causative gene is known. As a result, there have been numerous applications of antisense oligodeoxynucleotide (ODN) activity for potential antiviral and anticancer applications.

Antisense ODNs offer the potential to block the expression of specific genes within cells. Despite numerous reports of apparent antisense inhibition of gene expression in cultured cells, only in a few cases has specific inhibition been rigorously demonstrated. In many studies, specificity has been inferred from the biological effects of antisense as compared to control ODNs, without measuring levels of target RNA or proteins to evaluate specificity. Unintended side-effects of antisense technology could potentially occur through a number of mechanisms.

The potential of oligonucleotides as modulators of gene expression is currently under intense investigation. Most of the efforts are focused on inhibiting the expression of targeted genes such as oncogenes or viral genes. The oligonucleotides are directed either against RNA (antisense oligonucleotides) or against DNA where they form triplex structures inhibiting transcription by RNA polymerase II. To achieve a desired effect, the oligonucleotides must promote a decay of the preexisting, undesirable protein by effectively preventing its formation de novo.

There is therefore a need for the development of new antisense methods that are more potent, reliable and specific than those used in previous studies.

SUMMARY OF THE INVENTION

Compositions and methods for activating the expression of a gene of interest is provided. The compositions are antisense masked expression cassettes which comprise a double stranded nucleotide sequence. A first strand comprises an armed expression cassette, i.e., an RNA molecule which codes for a protein of interest linked downstream of a flanking sequence and a translation initiation site operably inserted upstream of the RNA sequence. The flanking sequence encodes a target molecule. That is, the flanking sequence encodes a target gene or codes for RNA of interest. The flanking sequence corresponds to the "sense" strand of the target. A second nucleotide strand is also provided, capable of hybridizing to the flanking sequence of the first nucleotide sequence; i.e., the antisense strand. The antisense strand masks the translation initiation site when bound. The flanking sequence can be designed so that the antisense sequences do not share 100% homology with the flanking sequence. Thus, in the presence of a target nucleotide molecule, the antisense strand will favor complementary binding with the target. In this manner, the antisense strand will disassociate from the armed strand and pair with the target. Disassociation of the antisense strand unmasks the ribosome binding site allowing the armed cassette to be translated in the presence of the target.

The compositions find use in regulation of gene expression, treatment of disease, and for preventing the proliferation of neoplastic cells. Additionally, the compositions have a broad range of use in both plant and animal applications.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a diagrammatic sketch of the masked targeted expression cassette as an antiviral drug.

Figure 2 provides a diagrammatic sketch of the masked targeted expression cassette in which the target sequence of the sense strand is completely complementary to the antisense strand.

Figure 3 provides a diagrammatic sketch of the masked expression cassette with concatenated geometry for increasing target specificity.

Figure 4 provides a diagrammatic sketch of the masked targeted expression cassette with concatenated geometry which requires a quantity threshold of target molecules for initiation of translation of the desired gene.

Figure 5 provides a diagrammatic sketch of a circular masked targeted expression cassette for increased compactness and decreased viscosity.

Figure 6 provides a diagrammatic sketch of a stem-loop masked targeted expression system for increased compactness.

Figure 7 provides an example of a construct for production of the sense strand of the targeted cassette.

Figure 8 provides a diagrammatic sketch of an *in vitro* experiment utilizing the masked targeted expression cassette.

DETAILED DESCRIPTION OF THE INVENTION

The present invention now will be described more fully hereinafter with reference to preferred embodiments. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will convey the scope of the invention to those skilled in the art.

Compositions and methods for controlling the expression of a gene of interest is provided. Expression is regulated by the use of antisense oligonucleotides to a target molecule. In this manner, the gene of interest is expressed only in the presence of RNA or DNA corresponding to the target molecule.

The method involves the use of an antisense masked expression cassette. By antisense masked expression cassette is intended a double stranded nucleic acid molecule. The first strand comprises an RNA molecule for the protein of interest linked downstream of a flanking sequence. The flanking sequence comprises a nucleotide sequence, the sense sequence, for a portion of the target gene. The first strand also comprises a translation initiation site downstream of the flanking sequence. It is recognized that the site of insertion for the ribosome binding site may vary. Optionally, a seven methyl guanine cap can be included to stabilize the molecule. See Figure 1.

The second strand of the masked expression cassette comprises an antisense oligonucleotide corresponding to the target gene or sequence. That is, the antisense sequence is at least partially complementary to the target sequence comprised by the flanking sequence. The antisense oligonucleotide may be either an RNA molecule, a DNA molecule or mixtures thereof. The duplex formed by binding of the second antisense strand to the corresponding flanking sequence of the first strand excludes ribosomal scanning of the downstream sequences; including the translation initiation site, the sequence of interest, or both. Thus, translation and expression of the protein of interest is masked by the binding of the antisense strand to the flanking sequence. Displacement of the antisense strand from the flanking sequence unmasks expression and translation of the protein of interest.

The protein of interest will vary depending upon the use of the composition. For example, where the masked cassette is intended as a mechanism to inhibit the growth of neoplastic cells, the protein of interest is selected from toxin proteins, cytokines, cell regulators, or the like. Additionally, the RNA molecule may be non-coding RNA, such as RNA with RNAse activity.

A number of toxin proteins are known and can be used in the invention. These include ribosome inactivators, Pseudomonas exotoxin A (Chaudhary et al. (1990) *J. Biol Chem 265*:16303-16310); cell metabolism disruptors, such as ribonucleases (See, for example, Mariani et al. (1990) *Nature 347*:737-741); Barnase toxin, a chimeric toxin derived from Pseudomonas exotoxin A and a ribonuclease (Prior et al. (1990), *Cell 64*:1017-1023); Pertussis (Accession M14378 M16494, Micosia *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:4631-4635); cholera (Dams *et al.* (1991) *Biochim. Biophys. Acta 1090*:139-141); Diphtheria, ricin (Gelfand *et al.* EP 0335476-A2); etc. Additionally, thymidine kinase from the herpes sequence may be used as a toxin or effector molecule. Transcription in a cell makes it susceptible to gancyclovir. Thus, the cells of interest could be labeled and then destroyed in a two step system.

The masked cassette comprising the above described toxin sequences can be used to target and destroy any cell, organ, organism or species; so long as a target sequence can be identified that is specific to that cell, organ, organism or species. For example, the cassette can be used to selectively target and eliminate vertebrate environmental pests. Such pests include agricultural pests including foxes and rabbits in Australia.

The cassette can be used to selectively destroy cells infected with viruses. In this aspect, the target sequence comprises a virus-specific sequence, while the protein of interest is a toxin as described above.

The masked cassette can be used to treat a variety of diseases. Such diseases include, but are not limited to, diseases involving an overactive organ, such as a hyperactive thyroid. In this aspect, the masked cassette comprises a thyroid-specific target sequence and a protein toxin as described above.

The cassette can be used to treat diseases involving a defective gene. In this aspect, the target sequence comprises the sequence of the defective mRNA, while the sequence of interest comprises the sequence of the normal protein. The intended affect can be twofold. The binding of the antisense strand to the defective mRNA can shut down the production of the defective protein, while expression of the sequence of interest results in production of the normal protein.

The cassette can be used to produce a protein of interest in an organ which lacks the protein. In this aspect, the target sequence comprises an organ-specific sequence, while the sequence of interest comprises the sequence of the protein lacking in that organ.

The invention is also useful in an assay system to determine the presence of a target molecule. In this instance the protein of interest will be a reporter protein that is easily detected, for example, by either a simple cytological stain or an enzyme assay. Such reporter sequences include but are not limited to beta galactosidase, chloramphenicol acetyltransferase (CAT), glucurodinase (GUS), and the like.

A translation initiation site is also included in the cassette. Such sequences are known in the art and include the Kozak sequence. See, for example, Kozak, Marilyn (1988) *Mol. and Cell Biol., 8*:2737-2744; Kozak, Marilyn (1991) *J. Biol. Chem., 266*:19867-19870; Kozak, Marilyn (1990) *Proc Natl. Acad. Sci. USA, 87*:8301-8305; Kozak, Marilyn (1989) *J. Cell Biol., 108*:229-241; and the references cited therein. Such references are herein incorporated by reference.

The translation initiation site can be inserted upstream of the sequence corresponding to the gene of interest. Kozak sequences can be designed that can initiate translation in all three reading frames. See, for example, Murphy and Efstratiadias (1987) *Proc. Natl. Acad. Sci. USA, 84*:8277-8281. Generally, the Kozak sequence will comprise the consensus sequence recognized for initiation in higher eukaryotes. Such consensus sequence is GCCGCC$^{A}_{G}$CCAUGG. This consensus sequence is repeated several times within the Kozak sequence to provide for the initiation of translation in all three reading frames.

The length of the Kozak sequence may vary. Generally, increasing the length of the leader sequence enhances translation.

It is recognized that a prokaryotic translation initiation site may also be used when appropriate; for example, when targeting a prokaryote. Such sequences include the Shine-Dalgarno sequence (UAAGGAGG), typically 5-10 bases upstream of the initiator AUG.

The flanking sequence comprises a sequence which corresponds to the target gene or sequence. That is, the flanking sequence comprises all or a part of the sense strand of the target molecule and can be RNA or DNA. By sense sequence is intended a sequence capable of hybridizing to the antisense portion capable of hybridizing to messenger RNA expressed by the target when the target is a gene, or to a target RNA or DNA molecule.

The flanking sequence may vary in length. It is recognized that the length may vary depending on the length and abundance of the target gene, and the specificity and affinity of the antisense portion for the target. While the length of the flanking sequence may vary, generally a length of about 10 to about 200 nucleotides, preferably about 20 to about 150 nucleotides, more preferably about 40 to about 100 nucleotides can be used.

The flanking sequence can be a naturally occurring or synthetic sequence. Where the sequence is synthetic, mismatch nucleotides can be incorporated into the structure to facilitate thermodynamic displacement of the antisense molecule by the target molecule. It is recognized that if the translation initiation site is inserted within the flanking sequence, this sequence insertion will provide nonhybridizing sequences and add to the decrease in homology between the flanking sequence and the antisense oligonucleotide. While it is recognized that a homology of up to 100% can be compatible with the intended displacement of the antisense strand from the flanking sequence, generally a homology of less than 90% is intended, preferably about 75% homology, more preferably about 65% homology.

A 7-methyl guanine (7MeG) cap is known to increase the efficiency of translation. Thus, such a 7-methyl guanine cap can be included on the 5' end of the flanking sequence. See, for example, Shatkin, A.J. (1976) *Cell, 9*:645-653; Malone *et al.* (1989) *Proc. Natl. Acad. Sci. USA, 86*:6077-6081; Fuerst and Moss (1989) *J. Mol. Biol., 206*:333-348 and Kozak, Marilyn (1991) *Gene Expression, 1*:117-125.

The antisense sequence of the expression cassette of the invention is constructed to hybridize with a nucleotide sequence of interest. Such nucleotide sequences of interest include messenger RNAs from target genes, viral RNAs or

DNAs, and the like. The antisense strand is constructed to be homologous to the target. Generally, such homology will be greater than the homology exhibited by the antisense strand to the flanking sequence. Thus, in the presence of the target molecule, the antisense strand is displaced from the flanking sequence of the cassette and hybridizes with the target molecule. To enhance displacement, the cassette can be constructed such that the antisense sequence is longer than the flanking sequence, allowing for a 3′ or 5′ nonpaired overhang or "sticky end" to bind the target molecule. This sticky end will enhance displacement of the antisense oligonucleotide.

As discussed, the target molecule may vary. For treatment of malignant or neoplastic cell growth, the target molecule will correspond to a nucleotide which is only expressed or present in the neoplastic cell. In this case, the sequence of interest of the expression cassette will encode a toxin protein which is expressed in the presence of the target to kill the cell. The expression cassette could also encode a cytokine or interferon to fight neoplastic growth. In some instances, a combination of expression cassettes encoding different proteins may be provided. The target molecule can be a gene. Numerous target genes are known in the art. Such genes include c-myc, n-myc, c-myb c-abl, c-kit, c-mos, bcr-abl, bcl-2, retinoblastoma-1, p-53, GM-CSF, G-CSF, Ick, IGF-1, egr-1 (A. Krieg, *ImmunoMethods* 1, 191 (1992)); c-fes (S. Ferrari et al., *Cell Growth Differ.* 1, 543 (1990)); c-fms (J. Wu et al., *Oncogene* 5, 873 (1990)); c-fos (A. Block et al., in (77). pp. 63-70); N-ras (T. Skorski et al., *J. Exp. Med.* 175, 743 (1992)); Ha-ras (T. Saison-Behmoaras et al., *EMBO J. MD.*, 1111 (1991)); B-myb (M. Arsura et al., *Blood* 79, 2708 (1992)); CSF-1 (M. Birchenall-Roberts et al., *J. Immunol.* 145, 3290 (1990)); Myeloblastin (D. Bories et al., *Cell* 59, 959 (1988)); Erythropoietin (O. Hermine et al., *Blood* 78, 2253 (1991)); MZF-1 (L. Bavisotto et al., *J. Exp. Med.* 174, 1097 (1991)); mdr1 (L. Rivoltini et al., *Int. J. Cancer* 46, 727 (1990)); IGF-1 receptor (P. Porcu et al., *Mol. Cell. Biol.* 12, 5069 (1992)); Growth hormone (D. Weingent, J. Blalock, R. LeBoeuff, *Endocrinology* 128, 2053 (1991)); EGR-1 (L. Neyses, J. Nouskas, H. Vetter, *Biochem. Biophys. Res. Commun.* 181, 22 (1991)); G proteins (Supra (1992)); MHC-1# (M. Cambe et al., *Anti-Cancer Drug Des.* 7, 341 (1992)); Angiotensinogen (J. Cook et al.,

Antisense Res. Dev. 2, 199 (1992); Myogenin (A. Brunetti et al., *J. Biol. Chem.* 265, 13435 (1990)); LH receptor** (A. West and B. Cooke, *Mol. Cell. Endocrinol.* 79, R9 (1991)); Cellular retinol-binding protein I F. Cope, J. Wille, L. D. Tomei, in (77), pp. 125-142; TNF-α (A. Witsell and L. Schook, *Proc. Natl. Acad. Sci. U.S.A.* 89, 4754 (1992)).

Target molecules include but are not limited to the CD4 gene, see, Accession No. X87579; CFTR gene (Varon *et al.* (1995) *Hum. Mol. Genet* *4*:1463-1464); human C3d/Epstein-Barr virus receptor (Fujisaku *et al.* (1989) *J. Biol. Chem. 264*:2118-2125); Human MHC class I CD8 alpha-chain gene (Accession M27161, Nakayama *et al.* (1989) *Immunogenetics 30*:393-397); human elastase 2 mRNA (Accession M16631), Fletcher *et al.* (1987) *Biochemistry 26*:7256-7261); Human elastin mRNA (Accession M36860, Fazio *et al.* (1988) *J. Invest. Dermatol. 91*:458-464); human intercellular adhesion molecule 1 gene (Accession U86814); human interleukin 1-beta converting enzyme isoform beta mRNA (Accession U13697 Alnemri *et al.* (1998) *J. Biol. Chem. 270*:4312-4317); human immunoglobulin C mu-C delta locus (Accession X57331, Word *et al.* (1989) *Int. Immunol 1*:296-309); human interleukin 2 gene (Accession J00264, Maeda *et al.* (1983) Biochem. *Biophys. Res. Commun. 115*:1040-1047; Fujita *et al.* (1983) *Proc. Natl. Acad. Sci. USA 80*:7437-7441); human MHC Class I antigen HLA-B (Accession U88407); human MHC class II HLA-DPA1 antigen (Accession U87556); etc. herein incorporated by reference.

Likewise, the target molecule may be a RNA or DNA from a virus. In this manner, viral replication and growth can be inhibited. Such viral genes include but are not limited to sequences from Coxsackievirus (Marquardt and Ohlinger (1995) *J. Virol. Methods 53*:189-199); Dengue virus, see Accession No. U88535; encephalitis virus, see, Accession No. AB001026; Ebola virus (Sanchez *et al.* (1989) *Virology 170:*81-91, Accession No. L11365); Epstein-Barr virus (Baer *et al.* (1984) *Nature 310*:207-211); Echovirus 32 (Huttunen *et al.* (1996) *J. Gen. Virol. 77*:715-725); Enterovirus (VP4-VP2 capsid 3D RNA polymerase genes Pulli *et al.* (1995) *Virology 212*:30-38); influenza A virus (Guan *et al.* (1996) *J. Virol. 70*:8041-8046); hepatitis B virus (Fukuda *et al.* (1995) *J. Infect. Dis. 172*:1191-1197); hepatitis C virus (Hitomi *et al.* (1995) *Viral Immunol.*

8:109-119); hepatitis D virus (Khudyakov *et al.* (1993) *Virus Res. 27*:13-24); hepatitis E virus (Tam *et al.* (1990) *Science 247*:1335-1449, Accession No. M32400); hepatitis G virus (Accession No. U86023); HIV (Accession U04908, Gao *et al.* (1996) *J. Virol. 70*:1651-1667); human papillomavirus (Accession U37537, Wu *et al.* (1993) *Lancet 341*:522-524); influenza A virus (Accession U86987); human rhinovirus (Accession D00239, Hughes *et al.* (1988)*J. Gen. Virol. 69*:49-58); Sendai virus (Accession D00053 N00053, Morgan and Rakestraw (1986) *Virology 154*:31-40); gastroenteritis virus TFI virion protein gene (Accession Z35758; Chen *et al.* (1995) *Virus Res. 38*:83-89); herpes simplex type 2 virus (Accession Z86099, McGeoch *et al.* (1987) *J. Gen Virol. 68*:19-38); Venezuelan equine encephalitis virus (Accession L01442, Kinney *et al.* (1986) *Virology 152*:400-413); herein incorporated by reference.

Other genes of interest include, for example, *jun*, bFGF, *wnt*-1, TGF-beta, *spi*-1 for cytomegalovirus; NDR, *c-erbB*-2 for herpes simplex virus, types 1 and 2; *bcl*-2 and *bci-abl* for human papilloma virus; p53 and *c-myb* for hepatitis, type B; l-*myc* and *ras* for influenza virus; etc.

Methods are generally available in the art for construction of the masked expression cassettes. See, for example, Sambrook *et al.*, Cold Spring Harbor, NY. RNA/DNA molecules as well as antisense oligonucleotides can be made in accordance with known techniques. See, e.g., U.S. Patent No. 5,149,797; 5,175,273; Uhlmann and Peyman (1990) *Chem. Rev., 90*:543-584 and the references cited therein. The antisense oligonucleotides, which may be deoxyribonucleotide or ribonucleotide sequences which are capable of complementary binding to the target molecule. Such antisense oligonucleotides may be oligonucleotides wherein at least one, or all, of the internucleotide bridging phosphate residues are modified phosphates, such as methyl phosphonates, methyl phosphonothioates, phosphoromorpholidates, phosphoropiperazidates and phosphoramidates. For example, some, for example, every other one, of the internucleotide bridging phosphate residues may be modified as described. In another example, such antisense oligonucleotides are oligonucleotides wherein at least one, or all, of the nucleotides contain a 2′ loweralkyl moiety (e.g., C1-C4, linear or branched, saturated or unsaturated alkyl,

such as methyl, ethyl, ethenyl, propyl, 1-propenyl, 2-propenyl, and isopropyl). See also Furdon *et al.* (1989) *Nucleic Acids Res., 17*:9193-9204; Agrawal *et al.* (1990) *Proc. Natl. Acad. Sci. USA, 87*:1401-1405; Baker *et al.* (1990) *Nucleic Acids Res., 18*:3537-3543; Sproat *et al.* (1989) *Nucleic Acids Res., 17*:3373-3389; Walder and Walder (1988) *Proc. Natl. Acad. Sci. USA, 85*:5011-5015.

Modification of the phosphodiester backbone has been shown to impart stability and may allow for enhanced affinity and increased cellular penetration of ODNs. Additionally, chemical strategies may be employed to replace the entire phosphodiester backbone with novel linkages. Phosphorothioate and methylphosphonate modified ODNs may be made through automated ODN synthesis.

A phosphorodithioate version of the phosphorothioate can be synthesized. In the dithioate linkage, the non-bridging oxygens can be substituted with sulfur. This linkage is highly nuclease resistant.

Sugar modifications may also be used to enhance stability and affinity of the molecules. The alpha-anomer of a 2'-deoxyribose sugar has the base inverted with respect to the natural beta-anomer. ODNs containing alpha-anomer sugars are resistant to nuclease degradation.

If necessary, targeted cassette can be modified to increase stability *in vivo*. Thus, nuclease-resistant oligonucleotides can be utilized, such as PS and MP oligonucleotides. See, for example, Miller, P. (1991) *Biotechnology, 9*:358 and Stein *et al.* (1991) *Pharmacol. Ther., 52*:365.

The targeted expression cassettes of the invention can be synthesized easily and in bulk. The development of phosphoramidite chemistry and its elaboration into an automated technology have greatly enhanced the ease with which oligos are synthesized and consequently their availability. See, for example, Beaucage and Caruthers (1981) *Tetrahedron Lett., 37*:3557 and Zon and Geiser (1991) *Anti-Cancer Drug Des., 6*:539.

The methods, oligonucleotides and formulations of the present invention have a variety of uses. They are useful in preventing the proliferation and growth of neoplastic cells. The methods, oligonucleotides and compositions of the present invention are also useful as therapeutic agents in the treatment of

disease. They also find use in fermentation processes where it is desirable to have a means for regulating the expression of a gene to be expressed at a certain time or any instance where it is desirable to regulate gene expression.

The term "antisense oligonucleotides" includes the physiologically and pharmaceutically acceptable salts thereof: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. Examples of such salts are (a) salts formed with cations such as sodium, potassium, spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, napthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, napthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

Formulations of the present invention comprise the masked cassette in a physiologically or pharmaceutically acceptable carrier, such as an aqueous carrier. Thus, formulations for use in the present invention include, but are not limited to, those suitable for parenteral administration, including subcutaneous, intradermal, intramuscular, intravenous and intraarterial administration, as well as topical administration (i.e., administration of an aerosolized formulation of respirable particles to the lungs of a patient afflicted with cystic fibrosis). The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art. Such formulations are described in, for example, *Remington's Pharmaceutical Sciences* 19th ed., Osol, A. (ed.), Mack Easton PA (1980). The most suitable route of administration in any given case may depend upon the subject, the nature and severity of the condition being treated, and the particular active compound which is being used.

The present invention provides for the use of the targeted masked cassette having the characteristics set forth above for the preparation of a medicament for the various disorders. In the manufacture of a medicament according to the

invention, the masked cassette is typically admixed with, *inter alia*, an acceptable carrier. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the patient. The carrier may be a solid or a liquid. One or more antisense oligonucleotides may be incorporated in the formulations of the invention, which may be prepared by any of the well known techniques of pharmacy consisting essentially of admixing the components, optionally including one or more accessory therapeutic ingredients.

Formulations of the present invention may comprise sterile aqueous and non-aqueous injection solutions of the active compound, which preparations are preferably isotonic with the blood of intended recipient and essentially pyrogen free. These preparations may contain anti-oxidants , buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions may include suspending agents and thickening agents. The formulations may be presented in unit dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or water-for-injection immediately prior to use.

In the formulation the targeted cassette may be contained within a lipid particle or vesicle, such as a liposome or microcrystal, which may be suitable for parenteral administration. The particles may be of any suitable structure, such as unilamellar or plurilamellar, so long as the targeted cassette is contained therein. Positively charged lipids such as N-[1-(2,3-dioleoyloxi)propyl]-N,N,N-trimethyl-amoniummethylsulfate, or "DOTAP," are particularly preferred for such particles and vesicles. The preparation of such lipid particles is well known. See, e.g., U.S. Patent Nos.4,880,635 to Janoff et al.; 4,906,477 to Kurono et al.; 4,911,928 to Wallach; 4,917,951 to Wallach; 4,920,016 to Allen et al.; 4,921,757 to Wheatley et al.; etc.

The dosage of the targeted cassette administered will depend upon the particular method being carried out, and when it is being administered to a subject, will depend on the disease, the condition of the subject, the particular formulation, the route of administration, etc. In general, intracellular

concentrations of the cassette of from .05 to 50μM, or more particularly .2 to 5 μM, are desired. For administration to a subject such as a human, a dosage of from about .01, .1, or 1 mg/Kg up to 50, 100, or 150 mg/Kg is employed.

Current technology has focused on antisense molecules only. Antisense oligonucleotides bind the offending RNA molecules in the cell. To be effective, a high dosage of antisense molecules have to be delivered to each cell. The present invention provides for an effector molecule which increases the potency of antisense technology. In this manner, the cell can be manipulated more easily and a far lower dosage, potentially even a 1 molecule to 1 cell ratio can be effective.

It is the idea of specificity that provides the underlying feature of the present invention. Standard cytotoxic chemotherapy for conditions such as neoplastic disease is fraught with systemic toxicity. The ratio of the toxic dose to the therapeutic dose is relatively low, which reflects the large number of cellular targets affected by the chemotherapeutic agent and the agents inability to distinguish between normal and diseased cells. In theory, this problem is solved by taking advantage of the specificity conferred by Watson-Crick base pair formation by identifying an appropriate target.

The following experiments are offered by way of illustration and not by way of limitation.

Experimental

Use of Targeted Cassette to Kill Neoplastic Cells

Following the protocols as essentially described above, a targeted cassette is constructed wherein the first strand has an RNA coding for toxin A. The toxin RNA is linked with upstream DNA sequences coding the sense portion of the p53 DNA sequence. The p53 protein is found in numerous cancer cells. Inserted within the p53 molecule is a Kozak sequence. An antisense structure is constructed which corresponds to the p53 sense nucleotide.

The targeted cassettes are provided to a patient in a pharmaceutically acceptable solution at a concentration of from about .01, .1, or 1 mg/Kg up to 50, 100, or 150 mg/Kg.

Use of Targeted Cassette as Antiviral Drug

Figure 1 depicts utilization of the masked targeted expression cassette as an antiviral agent (Black RNA Drug). Features of the cassette are identified by the provided key. The sense strand has a 7meG cap at its 5' end. In the inactive form, the antisense strand is hybridized to the flanking sequences of the sense strand; such that the Kozak sequence is masked. Mismatch area between the Kozak sequence and the antisense strand is indicated by lack of hydrogen bonding. Upon presentation of active viral RNA which has perfect homology to the antisense strand, the antisense strand dissociates from the sense strand and binds the viral RNA, which renders the viral RNA inactive. Furthermore, upon dissociation of the antisense strand, the Kozak sequence is unmasked and translation of the toxin protein commences from the AUG initiation codon. Upon production of toxic quantities of mature toxin, the cell hosting the virus is destroyed.

Targeted Cassette with Totally Complementary Antisense and Flank Sequences

Figure 2 depicts a masked targeted expression cassette in which the viral target sequence of the sense strand is completely complementary to the antisense strand. In the inactive targeted cassette, ribosomal assembly and scanning from the 5' end is prevented by the duplex between the antisense strand and the flanking sequence. In this example, displacement of the antisense strand and activation of expression of the gene of interest (lac Z) can be tested by assaying for β-galactosidase activity.

Targeted Cassette with Increased Target Specificity

Figure 3 depicts a masked targeted expression cassette with concatenated geometry for increasing target specificity for initiation of translation of the gene of interest. Each antisense/sense combination (1-3) corresponds to a different target sequence. For example, 1 corresponds to a viral RNA, 2 corresponds to a cytokine RNA, and 3 corresponds to a host specific protein. RNA encoding the protein of interest is only expressed when all 3 target sequences are present in the target cell, effecting displacement of all 3 antisense sequences from the sense

sequences of the cassette, thereby allowing ribosomal assembly and scanning from the 5' end to proceed to the Kozak sequence and the AUG start codon.

Targeted Cassette with Target Quantity Threshold

Figure 4 depicts a masked targeted expression cassette with concatenated geometry for requiring a target quantity threshold for initiation of translation. All sense/antisense combinations (1-3) correspond to the same target RNA. RNA encoding the protein of interest is only expressed when the target RNA is present in sufficient quantity (in this example 3 copies) in the target cell, effecting displacement of all 3 antisense sequences from the sense sequences of the cassette, thereby allowing ribosomal assembly and scanning from the 5' end to proceed to the Kozak sequence and the AUG start codon. The concatenated geometry thus requires a threshold quantity of the target RNA for initiation of translation. Such concatenated cassette constructs are particularly useful for targeting cancer cells with abnormally high number of copies of a particular mRNA. The constructs may also be made in combination with the constructs of Figure 3.

Circular Targeted Cassette

Figure 5 depicts a circular masked targeted expression cassette.

An antisense molecule complementary to a target molecule is bound to complementary sequences at the 3' and 5' end of the targeted expression cassette, thereby preventing ribosome assembly and scanning from the 5' end. Thus, displacement of the antisense strand in the presence of a complementary target molecule allows for translation and expression of the desired protein. The circular configuration may be more compact and less viscous, thereby having particularly desirable properties for drug delivery applications.

Stem-loop Targeted Cassette

Figure 6 depicts a stem-loop masked targeted expression cassette. An antisense molecule complementary to a target molecule is bound to complementary sequences within the loop structure, further stabilizing the stem-

loop structure. Ribosomal scanning from the 5' end is prevented from proceeding to the Kozak sequence and the initiation AUG codon by the stable secondary structure of the complex. Displacement of the antisense strand in the presence of a complementary target molecule provides a less stable stem-loop structure unable to prevent ribosomal scanning commenced from the 5' end.

Figure 7: Armed Sense Strand Plasmid Construct.

Figure 7 depicts a $_{P}$CI-Neo (Promega Corp.) plasmid construct for production of the sense RNA strand of a targeted expression cassette. The complete sequence of the depicted MCS-Kozak-lac Z is set forth in SEQ ID NO: 1. Alternative flanking sequences corresponding to portions of the firefly luciferase mRNA are inserted into the multiple cloning site (MCS), such that transcription from the T_7 promoter yields RNA comprising from the 5' end; luciferase segment-Kozak-β gal. The alternative luciferase sense segments are set forth in SEQ ID NO: 2, 3, 4, 5, 6, 7 or 8.

Figure 8 *In vitro* determination of activity of masked targeted expression cassette.

Sense strand RNA of the masked targeted cassette is produced by *in vitro* transcription of the construct depicted in Figure 7, by use of the Riboprobe® Combination System (Catalogue No. P1450, Promega Corp.).

Antisense sequences corresponding to portions of the target molecule (firefly luciferase RNA, Catalogue No. L4561, Promega Corp.) are hybridized to complementary flanking sequences of the sense strand of the targeted cassette. SEQ ID NO: 2, 3, 4, 5, 6, 7 or 8 list alternative flanking sequences and SEQ ID NO: 9, 10, 11, 12, 13, 14 or 15 list the corresponding antisense sequences, respectively. The full length firefly luciferase RNA, according to which the flanking sense sequences and corresponding antisense sequences are made is set forth in SEQ ID NO: 16. The hybridized mixture is introduced to an *in vitro* translation mixture containing ribosomes and full length firefly luciferase RNA (Flex®, Rabbit Reticulocyte Lysate System, Catalogue No. L4540, Promega Corp.). Control reactions will lack the masked cassette.

After completion of translation, the mixture will be assayed for β-galactosidase (β-gal) and luciferase activities. Negative luciferase and positive β-gal activity indicates successful inhibition of the target molecule and successful expression of the gene of interest.

The diagram depicts the mechanism of the assay. In panel 1, the antisense sequence is bound to complementary flanking sequence of the targeted cassette. Ribosomal scanning commenced from the 5' end is blocked by the antisense/sense duplex, thereby preventing translation of the β-gal RNA. Displacement and binding of the antisense to target luciferase RNA (panel 2) has a two-fold effect (panel 3). β-gal can be expressed from the unmasked cassette (β-gal positive) and expression of the target is blocked by binding of the antisense to the target (luciferase negative).

Other modifications and embodiments of the invention will come to mind in one skilled in the art to which this invention pertains having the benefit of the teachings presented herein. Therefore, it is to be understood that the invention is not to be limited to the specific embodiments disclosed. Although specific terms are employed, they are used in generic and descriptive sense only and not for purposes of limitation, and that modifications and embodiments are intended to be included within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Black Jr., Charles A.

(ii) TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR ACTIVATING GENES OF INTEREST

(iii) NUMBER OF SEQUENCES: 16

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: W. Murray Spruill
(B) STREET: 3605 Glenwood Ave. Suite 310
(C) CITY: Raleigh
(D) STATE: NC
(E) COUNTRY: US
(F) ZIP: 27622

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Spruill, W. Murray
(B) REGISTRATION NUMBER: 32,943
(C) REFERENCE/DOCKET NUMBER: 5722-2

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 919 420 2202
(B) TELEFAX: 919 881 3175

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4279 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Recombinant molecule (Multiple Cloning Site/Kozack sequence/LacZ gene)"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..64
(D) OTHER INFORMATION: /product= "Multiple Cloning Site"

(ix) FEATURE:
(A) NAME/KEY: misc_feature

(B) LOCATION: 65..79
(D) OTHER INFORMATION: /function= "Consensus sequence of translation initiation"
/product= "Kozack sequence"

(ix) FEATURE:
(A) NAME/KEY: prim_transcript
(B) LOCATION: 80..4279
(D) OTHER INFORMATION: /gene= "LacZ"
/standard_name= "Beta galactosidase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TGGTTAGGTA CCTTCTGAGG CGGAAAGAAC CAGCTGTGGA ATGTGTGTCA GTTAGGGTGT 180

GGAAAGTCCC CAGGCTCCCC AGCAGGCAGA AGTATGCAAA GCATGCATCT CAATTAGTCA 240

GCAACCAGGT GTGGAAAGTC CCCAGGCTCC CCAGCAGGCA GAAGTATGCA AAGCATGCAT 300

CTCAATTAGT CAGCAACCAT AGTCCCGCCC CTAACTCCGC CCATCCCGCC CCTAACTCCG 360

CCCAGTTCCG CCCATTCTCC GCCCCATGGC TGACTAATTT TTTTTATTTA TGCAGAGGCC 420

GAGGCCGCCT CGGCCTCTGA GCTATTCCAG AAGTAGTGAG GAGGCTTTTT TGGAGGCCTA 480

GGCTTTTGCA AAAAGCTTGG GATCTCTATA ATCTCGCGCA ACCTATTTTC CCCTCGAACA 540

CTTTTTAAGC CGTAGATAAA CAGGCTGGGA CACTTCACAT GAGCGAAAAA TACATCGTCA 600

CCTGGGACAT GTTGCAGATC CATGCACGTA AACTCGCAAG CCGACTGATG CCTTCTGAAC 660

AATGGAAAGG CATTATTGCC GTAAGCCGTG GCGGTCTGGT ACCGGTGGGT GAAGACCAGA 720

AACAGCACCT CGAACTGAGC CGCGATATTG CCCAGCGTTT CAACGCGCTG TATGGCGAGA 780

TCGATCCCGT CGTTTTACAA CGTCGTGACT GGGAAAACCC TGGCGTTACC CAACTTAATC 840

GCCTTGCAGC ACATCCCCCT TTCGCCAGCT GGCGTAATAG CGAAGAGGCC CGCACCGATC 900

GCCCTTCCCA ACAGTTGCGC AGCCTGAATG GCGAATGGCG CTTTGCCTGG TTTCCGGCAC 960

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CATTTAATGT TGATGAAAGC TGGCTACAGG AAGGCCAGAC GCGAATTATT TTTGATGGCG 1200

TTAACTCGGC GTTTCATCTG TGGTGCAACG GGCGCTGGGT CGGTTACGGC CAGGACAGTC 1260

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TCATGGATGA GCAGACGATG GTGCAGGATA TCCTGCTGAT GAAGCAGAAC AACTTTAACG 1920

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TCAGTGTGAC GCTCCCCGCC GCGTCCCACG CCATCCCGCA TCTGACCACC AGCGAAATGG 3000

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CAAACTCATC AATGTATCTT ATCATGTCTG GATCCTCTAG AGTCGACCTG CAGGCATGCA 4260

AGCTGGCACT GGCCGTCGT 4279

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAATACAAAG CTTATGCATG 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATACAAAG CTT 13

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAAGCTTATG CATGCGGCCG 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGGCCGCATC TAGAGGGCCC 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGGCCGCAT CTAGAGGGCC CGGAT 25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AATACAAAGC TTATGCATGC GGCC 24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AATACAAAGC TTATGCATGC GGCCGCATCT 30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CATGCATAAG CTTTGTATTC 20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGCTTTGTA TTC 13

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGGCCGCATG CATAAGCTTT 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGGCCCTCTA GATGCGGCCG 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATCCGGGCCC TCTAGATGCG GCCGC 25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGCCGCATGC ATAAGCTTTG TATT 24

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGATGCGGCC GCATGCATAA GCTTTGTATT 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1798 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GAAUACAAAG CUUAUGCAUG CGGCCGCAUC UAGAGGGCCC GGAUCCAAAU GGAAGACGCC 60

AAAAACAUAA AGAAAGGCCC GGCGCCAUUC UAUCCUCUAG AGGAUGGAAC CGCUGGAGAG 120

CAACUGCAUA AGGCUAUGAA GAGAUACGCC CUGGUUCCUG GAACAAUUGC UUUUACAGAU 180

GCACAUAUCG AGGUGAACAU CACGUACGCG GAAUACUUCG AAAUGUCCGU UCGGUUGGCA 240

GAAGCUAUGA AACGAUAUGG GCUGAAUACA AAUCACAGAA UCGUCGUAUG CAGUGAAAAC 300

UCUCUUCAAU UCUUUAUGCC GGUGUUGGGC GCCGUUAUUU AUCGGAGUUG CAGUUGCGCC 360

CGCGAAGCAC AUUUAUAAUG AACGUGAAUU GCUCAACAGU AUGAACAUUU CGCAGCCUAC 420

CGUAGUGUUU GUUUCCAAAA AGGGGUUGCA AAAAAUUUUG AACGUGCAAA AAAAAUUACC 480

AAUAAUCCAG AAAAUUAUUA UCAUGGAUUC UAAAACGGAU UACCAGGGAU UUCAGUCGAU 540

GUACACGUUC GUCACAUCUC AUCUACCUCC CGGUUUUAAU GAAUACGAUU UUGUACCAGA 600

GUCCUUUGAU CGUGACAAAA CAAUUGCACU GAUAAUGAAU UCCUCUGGAU CUACUGGGUU 660

ACCUAAGGGU GUGGCCCUUC CGCAUAGAAC UGCCUGCGUC AGAUUCUCGC AUGCCAGAGA 720

UCCUAUUUUU GGCAAUCAAA UCAUUCCGGA UACUGCGAUU UUAAGUGUUG UUCCAUUCCA 780

UCACGGUUUU GGAAUGUUUA CUACACUCGG AUAUUUGAUA UGUGGAUUUC GAGUCGUCUU 840

AAUGUAUAGA UUUGAAGAAG AGCUGUUUUU ACGAUCCCUU CAGGAUUACA AAAUUCAAAG 900

UGCGUUGCUA GUACCAACCC UAUUUUCAUU CUUCGCCAAA AGCACUCUGA UUGACAAAUA 960

CGAUUUAUCU AAUUUACACG AAAUUGCUUC UGGGGGCGCA CCUCUUUCGA AAGAAGUCGG 1020

GGAAGCGGUU GCAAAACGCU UCCAUCUUCC AGGGAUACGA CAAGGAUAUG GGCUCACUGA 1080

GACUACAUCA GCUAUUCUGA UUACACCCGA GGGGGAUGAU AAACCGGGCG CGGUCGGUAA 1140

AGUUGUUCCA UUUUUUGAAG CGAAGGUUGU GGAUCUGGAU ACCGGGAAAA CGCUGGGCGU 1200

UAAUCAGAGA GGCGAAUUAU GUGUCAGAGG ACCUAUGAUU AUGUCCGGUU AUGUAAACAA 1260

UCCGGAAGCG ACCAACGCCU UGAUUGACAA GGAUGGAUGG CUACAUUCUG GAGACAUAGC 1320

UUACUGGGAC GAAGACGAAC ACUUCUUCAU AGUUGACCGC UUGAAGUCUU UAAUUAAAUA 1380

CAAAGGAUAU CAGGUGGCCC CCGCUGAAUU GGAAUCGAUA UUGUUACAAC ACCCCAACAU 1440

CUUCGACGCG GGCGUGGCAG GUCUUCCCGA CGAUGACGCC GGUGAACUUC CCGCCGCCGU 1500

UGUUGUUUUG GAGCACGGAA AGACGAUGAC GGAAAAAGAG AUCGUGGAUU ACGUCGCCAG 1560

UCAAGUAACA ACCGCGAAAA AGUUGCGCGG AGGAGUUGUG UUUGUGGACG AAGUACCGAA 1620

AGGUCUUACC GGAAAACUCG ACGCAAGAAA AAUCAGAGAG AUCCUCAUAA AGGCCAAGAA 1680

GGGCGGAAAG UCCAAAUUGU AAAAUGUAAC UGUAUUCAGC GAUGACGAAA UUCUUAGCUA 1740

UUGUAAUCCU CCGAGGGGGC GAGCUCCCAA AAAAAAAAAA AAAAAAAAAA AAAAAAAA 1798

CLAIMS:

1. A masked expression cassette comprising a double stranded nucleic acid molecule wherein a first strand comprises an RNA sequence which codes for a protein of interest linked downstream of a flanking sequence, and a translation initiation site operably inserted upstream of the RNA sequence; and,

a second antisense strand bound to the flanking sequence wherein said second strand corresponds to an antisense oligonucleotide to a target molecule.

2. The cassette of claim 1, wherein said cassette further comprises a 7-methyl guanine cap linked to the 5′ end of the flanking sequence.

3. The cassette of claim 1, wherein said protein of interest encodes a toxin.

4. The cassette of claim 1, wherein said target comprises an oligonucleotide which is unique to neoplastic cells.

5. A method for inhibiting the growth of neoplastic cells, said method comprising contacting said cells with a masked expression cassette comprising a double stranded nucleic acid molecule;

wherein a first strand comprises an RNA sequence which codes for a protein of interest linked downstream of a flanking sequence, and a translation initiation site operably inserted upstream of the RNA sequence; and,

a second antisense strand bound to the flanking sequence, wherein said second strand corresponds to an antisense oligonucleotide to a target molecule.

6. The method of claim 5, wherein said cassette further comprises a 7-methyl guanine cap linked to the 5′ end of the flanking sequence.

7. The method of claim 5, wherein said translation initiation site comprises a Kozak sequence.

8. The method of claim 5, wherein said protein of interest is a toxin.

9. The method of claim 8, wherein said target comprises a nucleotide sequence which is unique to neoplastic cells.

10. A method for controlling the expression of a protein of interest in the presence of a target molecule, said method comprising contacting a cell comprising the target molecule with a marked expression cassette comprising a double stranded nucleic acid molecule, wherein a first strand comprises an RNA sequence which codes for a protein of interest linked downstream of a flanking sequence, and a translation initiation site operably inserted upstream of the RNA sequence; and,

a second antisense strand bound the flanking sequence wherein said second strand corresponds to an antisense oligonucleotide to a target molecule.

11. The method of claim 10, wherein said cassette further comprises a 7-methyl guanine cap linked to the 5′ end of the flanking sequence.

12. The method of claim 10, wherein said protein of interest encodes a toxin.

13. The method of claim 10, wherein said target comprises a nucleotide sequence which is unique to neoplastic cells.

14. A method for producing a protein of interest in a specific organ, said method comprising contacting cells of said organ with a masked expression cassette comprising a double stranded nucleic acid molecule, wherein a first strand comprises an RNA sequence which codes for said protein of interest linked downstream of a flanking sequence, and a translation initiation site operably inserted upstream of the RNA sequence; and,

a second antisense strand bound to the flanking sequence wherein said antisense strand corresponds to a target molecule specific to said organ.

15. The method of claim 14, wherein said cassette further comprises a 7-methyl guanine cap linked to the 5' end of the flanking sequence.

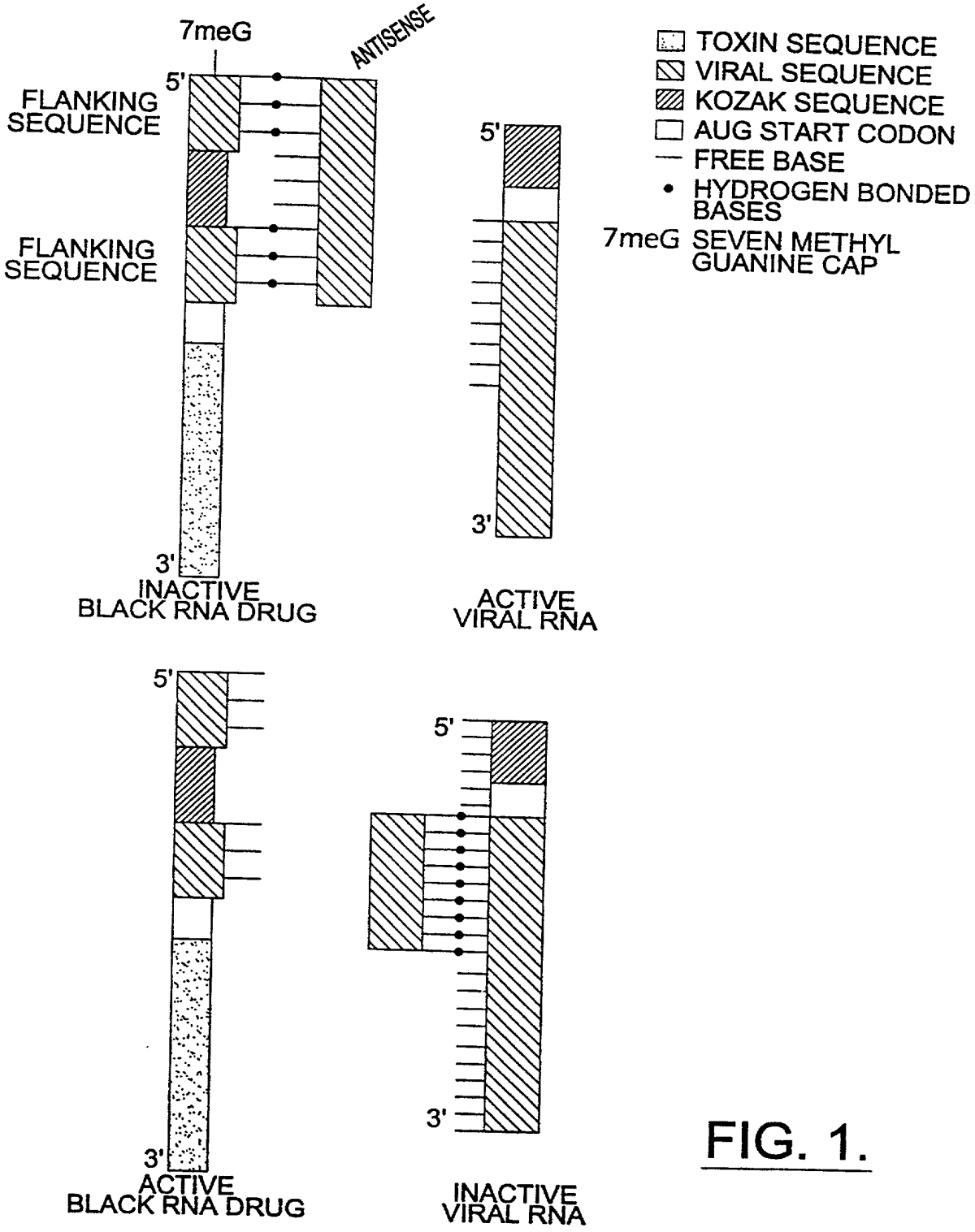
7meG
ANTISENSE
5'
FLANKING SEQUENCE
FLANKING SEQUENCE
3'
INACTIVE BLACK RNA DRUG
5'
3'
ACTIVE VIRAL RNA
TOXIN SEQUENCE
VIRAL SEQUENCE
KOZAK SEQUENCE
AUG START CODON
FREE BASE
HYDROGEN BONDED BASES
7meG SEVEN METHYL GUANINE CAP
5'
3'
ACTIVE BLACK RNA DRUG
5'
3'
INACTIVE VIRAL RNA

FIG. 1.

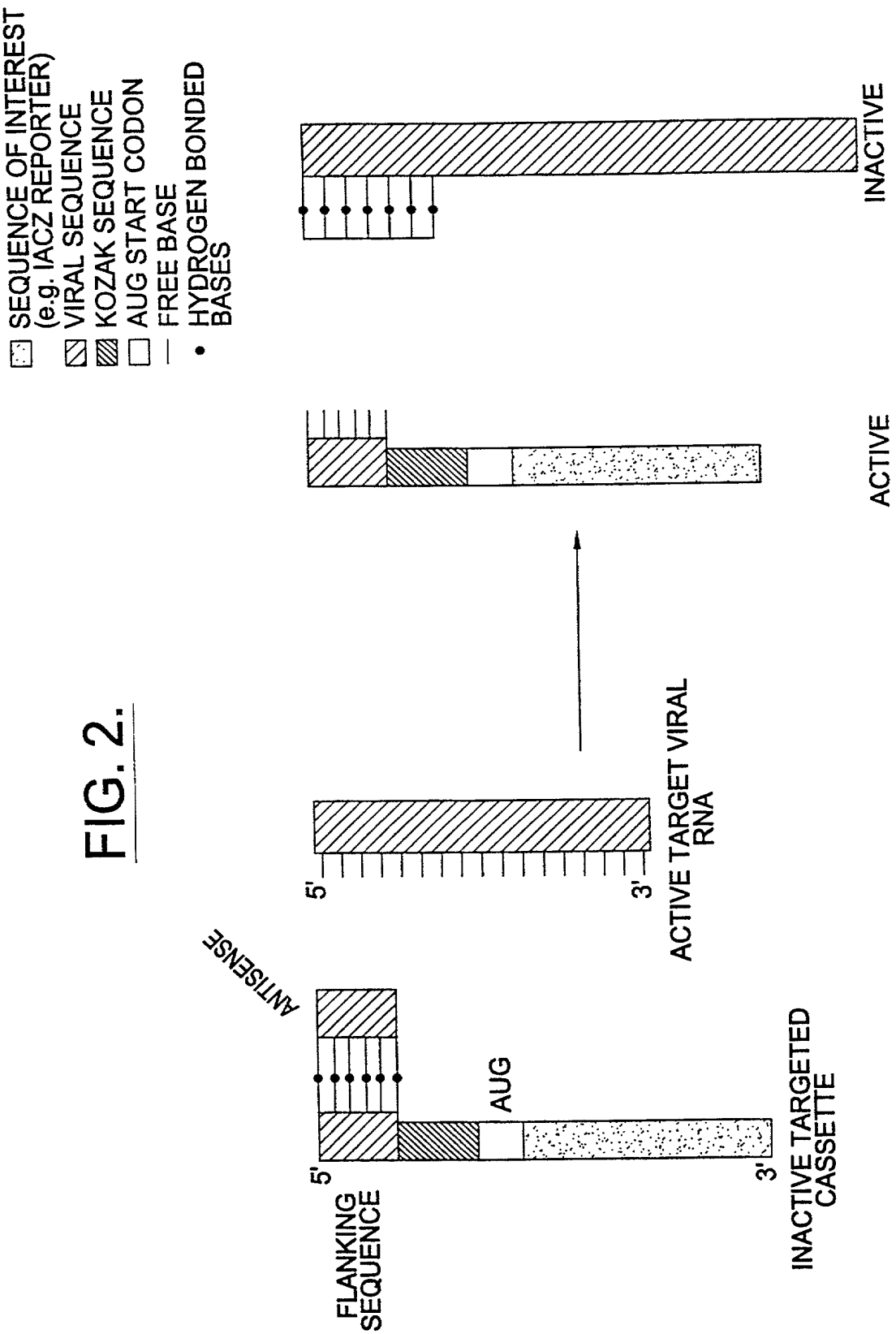
SEQUENCE OF INTEREST (e.g. IACZ REPORTER)
VIRAL SEQUENCE
KOZAK SEQUENCE
AUG START CODON
FREE BASE
HYDROGEN BONDED BASES
INACTIVE
ACTIVE
ACTIVE TARGET VIRAL RNA
5'
3'
ANTISENSE
FLANKING SEQUENCE
AUG
INACTIVE TARGETED CASSETTE

FIG. 2.

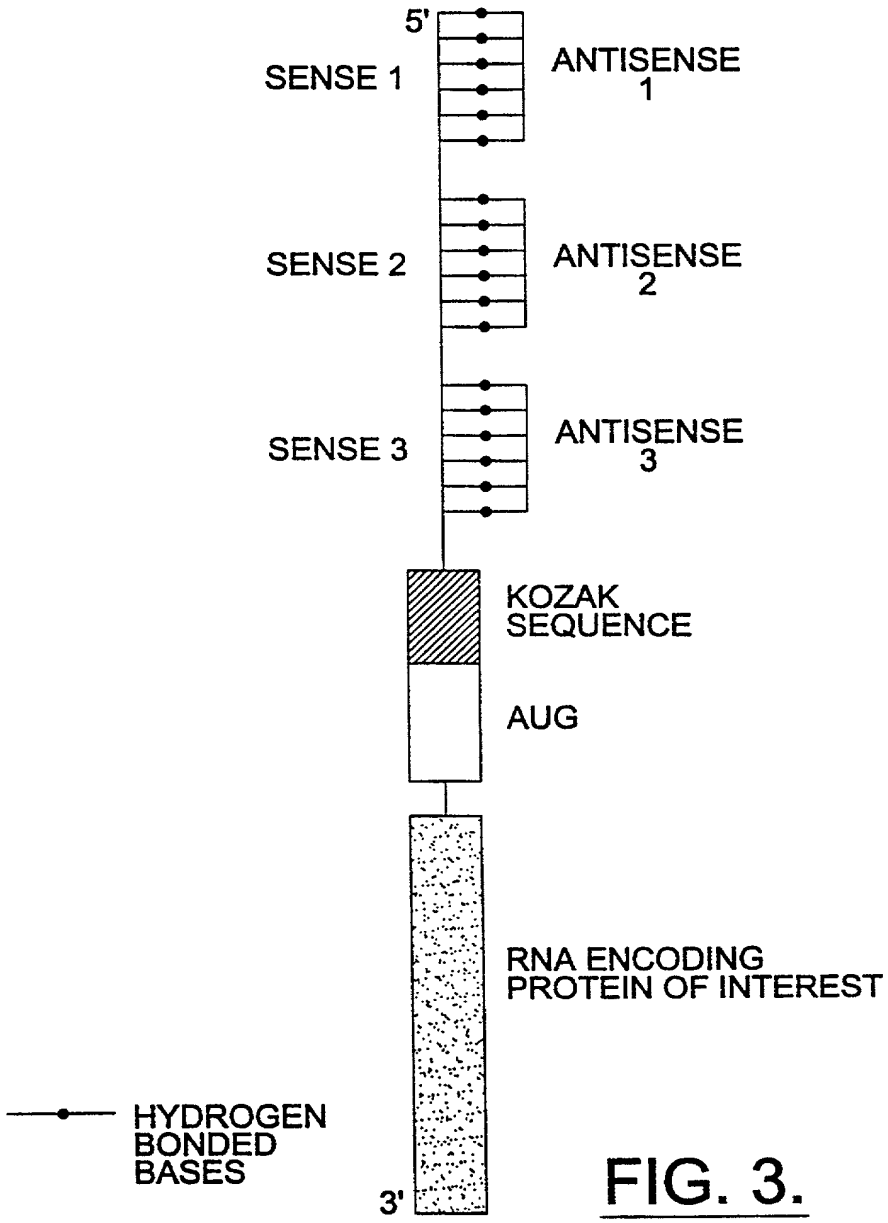
5'
SENSE 1
ANTISENSE 1
SENSE 2
ANTISENSE 2
SENSE 3
ANTISENSE 3
KOZAK SEQUENCE
AUG
RNA ENCODING PROTEIN OF INTEREST
3'
HYDROGEN BONDED BASES

FIG. 3.

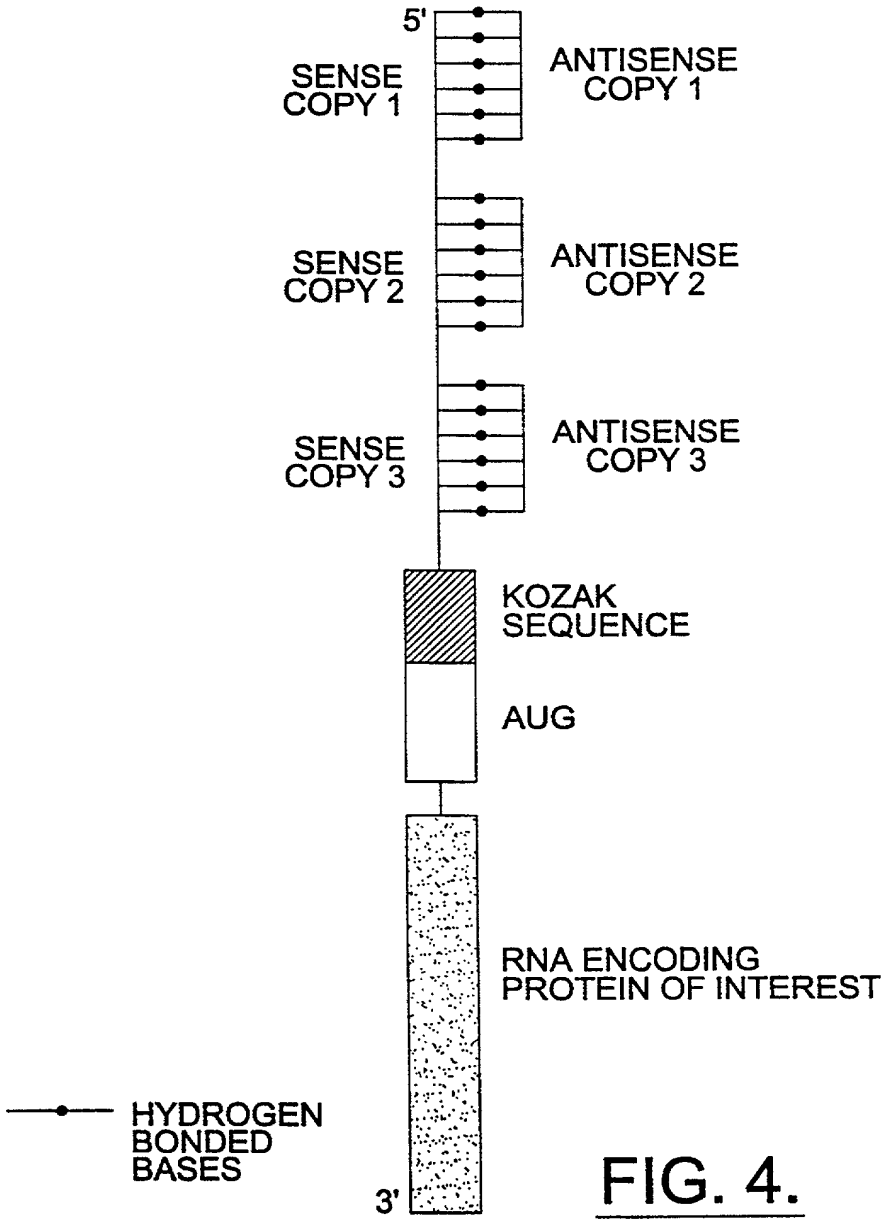
5'
SENSE COPY 1
ANTISENSE COPY 1
SENSE COPY 2
ANTISENSE COPY 2
SENSE COPY 3
ANTISENSE COPY 3
KOZAK SEQUENCE
AUG
RNA ENCODING PROTEIN OF INTEREST
3'
HYDROGEN BONDED BASES

FIG. 4.

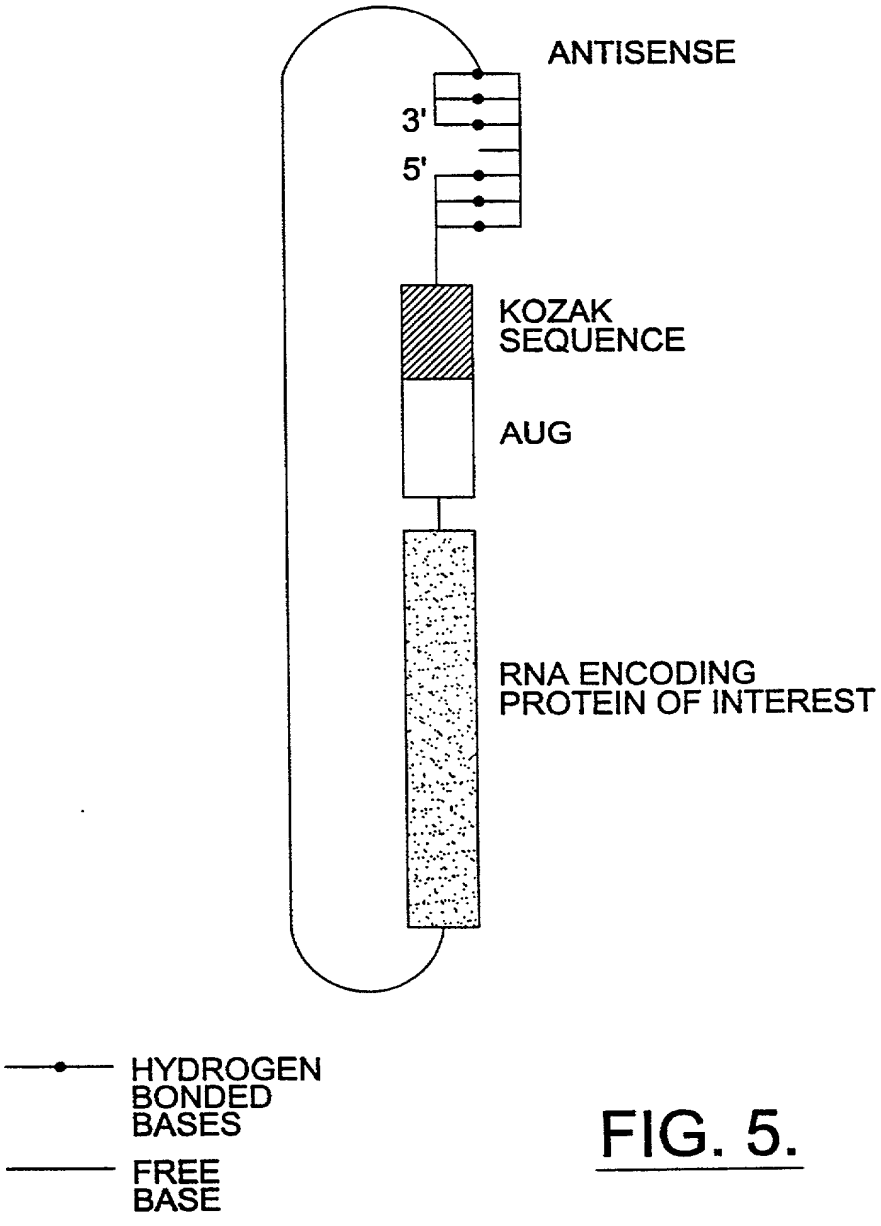
ANTISENSE
3'
5'
KOZAK SEQUENCE
AUG
RNA ENCODING PROTEIN OF INTEREST
HYDROGEN BONDED BASES
FREE BASE

FIG. 5.

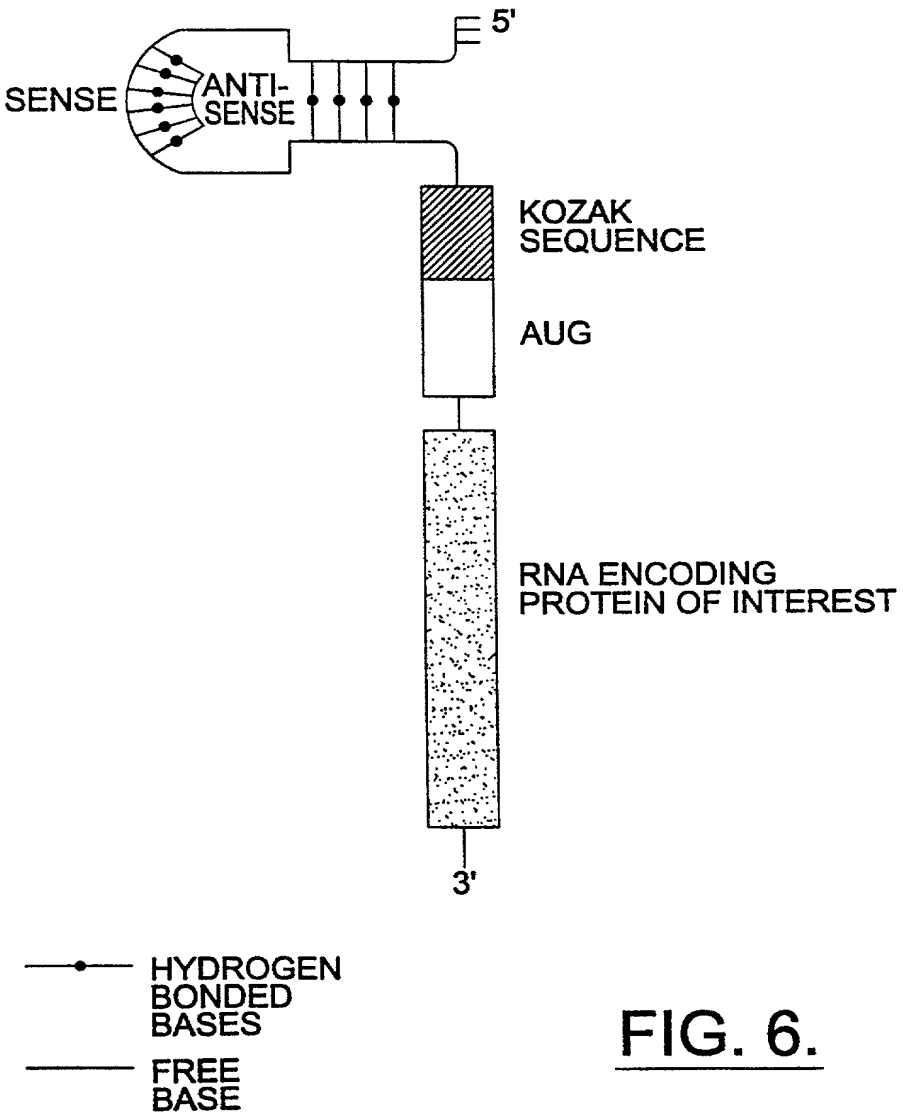
5'
SENSE
ANTI-
SENSE
KOZAK
SEQUENCE
AUG
RNA ENCODING
PROTEIN OF INTEREST
3'
HYDROGEN
BONDED
BASES
FREE
BASE

FIG. 6.

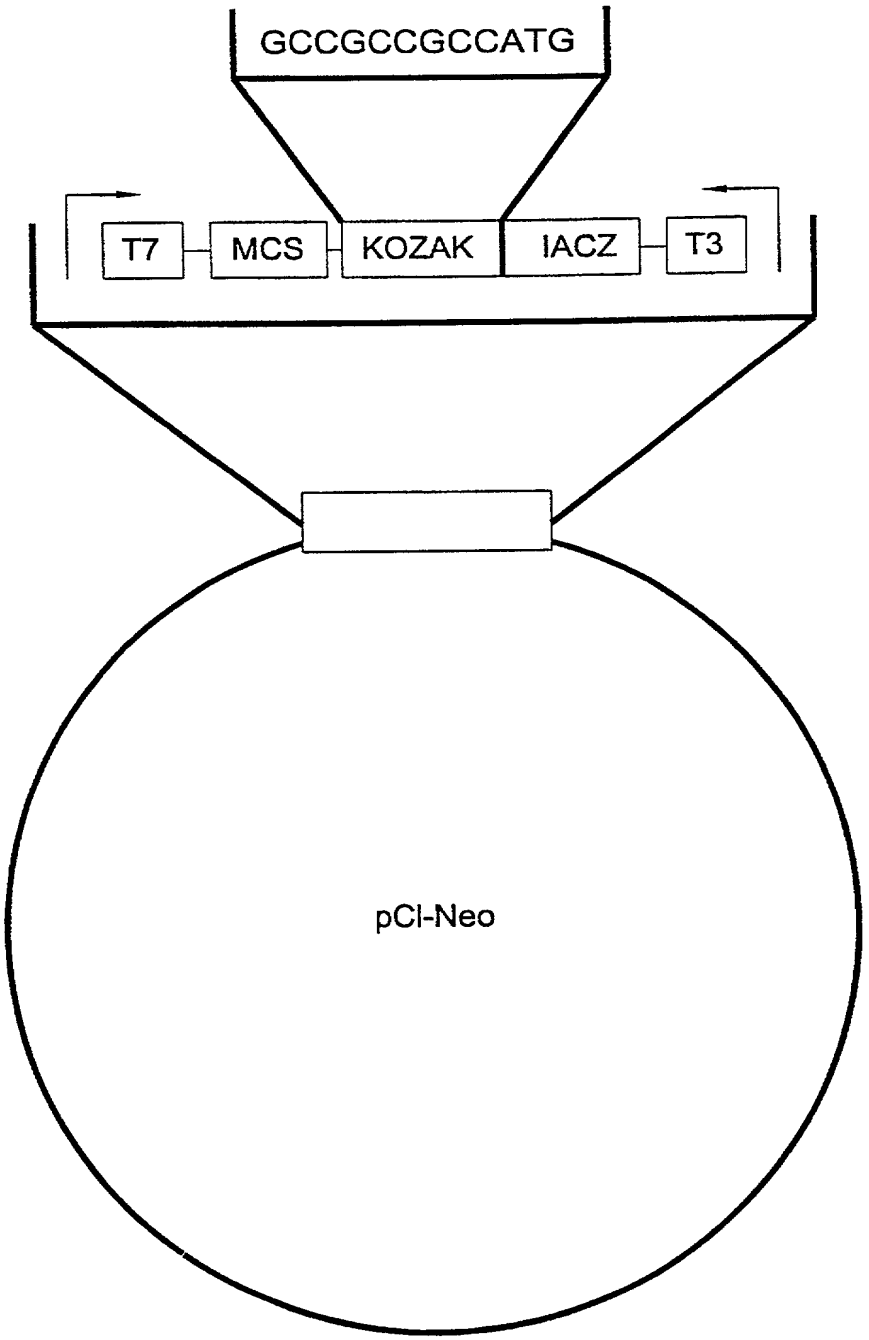

FIG. 7.

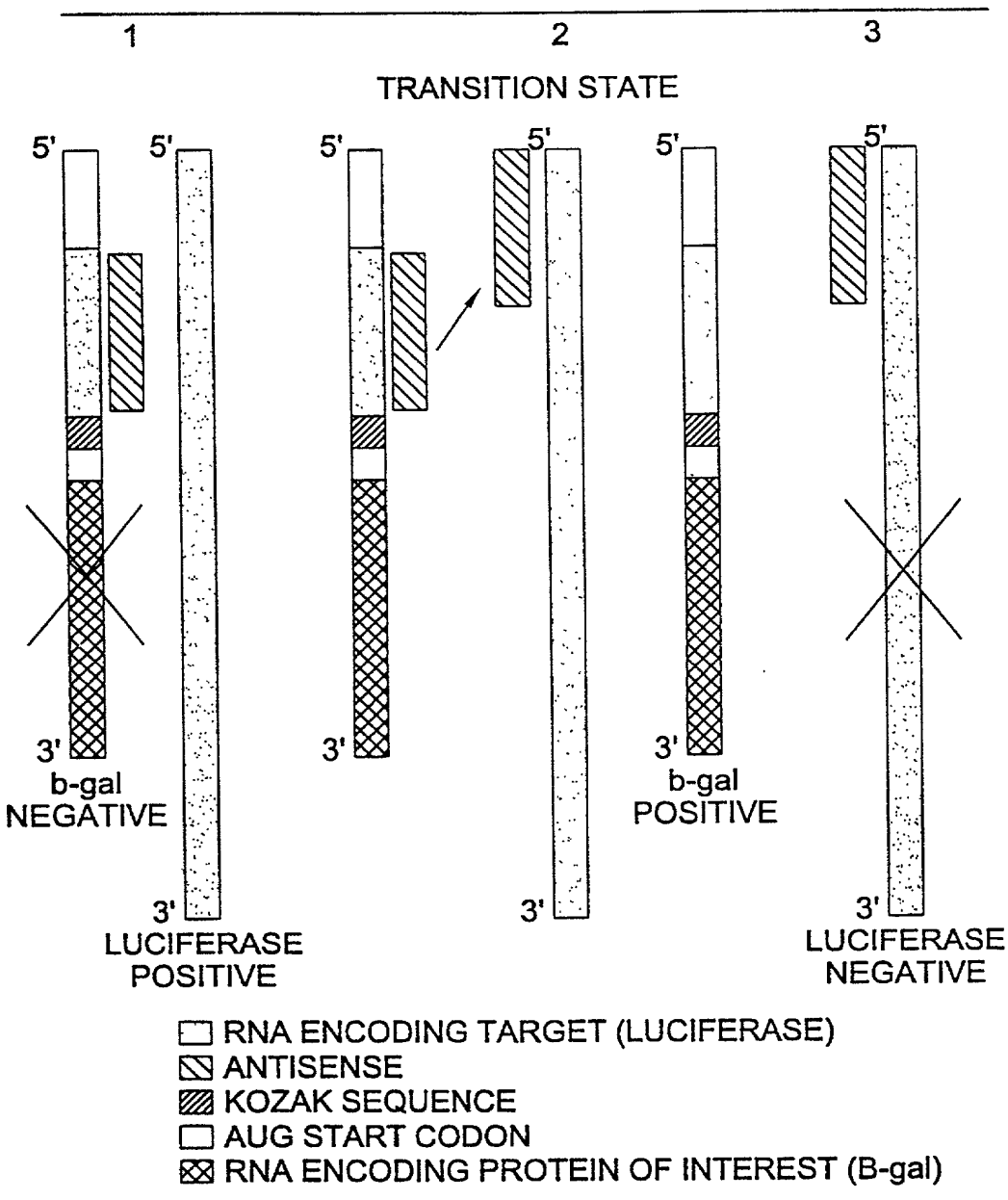
1
2
3
TRANSITION STATE
5'
3'
b-gal
NEGATIVE
LUCIFERASE
POSITIVE
b-gal
POSITIVE
LUCIFERASE
NEGATIVE
RNA ENCODING TARGET (LUCIFERASE)
ANTISENSE
KOZAK SEQUENCE
AUG START CODON
RNA ENCODING PROTEIN OF INTEREST (B-gal)

FIG. 8.

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)	Attorney Docket No.: 5722-2
	First Named Inventor: Charles Allen Black, Jr
	COMPLETE IF KNOWN
	Application Number: To be assigned
	Filing Date: Concurrently herewith
	Group Art Unit:
	Examiner Name:

As a below named Inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

COMPOSITIONS AND METHODS FOR ACTIVATING GENES OF INTEREST

the specification of which

☐ is attached hereto

OR

☒ was filed on June 24, 1998 as PCT International Application Number PCT/US98/13093 and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 (a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365 (a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				Yes	No
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	☐ Additional provisional application numbers are listed on a supplemental priority data sheet attached hereto.
60/050,772	06/25/97	

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1..56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*
PCT/US98/13093	06/24/98	

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto

As a named inventor, I hereby appoint the practitioners associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

Customer Number 000826

Direct correspondence to the attention of and telephone calls to:

W. Murray Spruill
Registration No. 32,943
ALSTON & BIRD LLP
Post Office Drawer 34009
Charlotte, NC 28234
Tel. Raleigh Office (919) 420-2200
Fax Charlotte Office (919) 420-2260

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

1-00

Full name of first inventor: Charles Allen Black, Jr.
Inventor's Signature: _____ Date: 11/21/94
Residence: Pittsburgh, Pennsylvania
Citizenship: United States of America
Post Office Address: 1139 Judy Ann Place
Pittsburgh, Pennsylvania

PA